IDRC Grant # 94-1050 / 01054

Immunologic Follow-up in High Titer Measles Vaccine Recipients in the Sudan

Final Report

Submitted by

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July 10, 1996

1. Summary

IDRC funded a trial of high titer (HT) measles vaccination in the Sudan from 1989-1992. This trial was designed to evaluate the effectiveness of high titer (Edmonston Zagreb or Connaught; EZ and CN respectively) vs standard titer Schwartz (SCH) measles vaccination (Phase I). The main study aims of Phase I were achieved but a second phase of investigation became necessary when other HT trials reported increased mortality associated with the higher titer vaccines. Dr Eric Hoskin's initial evaluation of the Sudan data suggested that a similar trend towards increased mortality in the HT recipients was also present in the Sudan trial. A site visit performed in 1993 suggested that a 5 year immunologic follow-up study in the Sudan site would be particularly important because:

1) The Sudanese children had been enrolled more recently (vs other study sites)
2) The trial was placebo controlled
3) The trial was unique in its collection of morbidity data
4) The study was unique in the use of high titer Connaught vaccine

A random sample of the original study participants was approached to seek their cooperation in the long-term immunologic follow-up. Over 3 months, 193 children were recruited (73 EZ, 61 CN, 59 SCH). Blood samples were collected for determination of antibodies directed against a variety of childhood pathogens. In addition, peripheral blood mononuclear cells (PBMC) were frozen for phenotypic analysis and *in vitro* tests of cellular immunity against childhood pathogens. Finally, the morbidity data collected over the first three years of the original study was analyzed to identify possible trends in morbidity associated with the HT vaccines.

The data we have generated can be summarized quickly and simply:

We performed more than 7,000 tests of immunologic function (> 35 tests/specimen) and found no consistent pattern of significant differences between the high and standard titer vaccine recipients. The mortality associated with HT vaccines was greater in females than males but we found no consistent differences between the sexes which could plausibly account for the excess mortality observed. Finally, no significant morbidity could be attributed to the HT vaccines in our review of more than 40,000 investigator visits over the 36 month period after vaccination.

These data are immensely reassuring since:

1) earlier studies had demonstrated subtle immunologic changes in HT recipients
2) > 20 million doses of the HT vaccines were distributed throughout the world before their use was suspended

These data suggest that the purported influence of HT vaccines on child survival is idiosyncratic rather than a general immunologic insult with a 'dose-response' effect on resistance to infection.

2. Summary in Lay Terms

Standard measles vaccine does not protect children before 9 months of age, a time when the complications of this infection may be particularly severe. In the late 1980's, measles vaccines containing increased doses of the vaccine virus were introduced in an attempt to protect these very young children. Although the vaccines worked well against measles, several large studies reported that recipients of the high titer vaccines were at increased risk of death from other illnesses for at least 2-3 years after vaccination. This increased risk of death was greatest in girls and several studies reported subtle differences in the immune systems of the high titer vaccine recipients up to $2^1/_2$ years after vaccination.

We performed a detailed study of the immune status of a subset of children enrolled in a trial of high tier measles vaccines in the Sudan 4-5 years after the suspect vaccination. We evaluated a wide variety of immunologic parameters such as antibody production and cellular recognition of microorganisms (immune memory) in these children. In all, we performed more than 7,000 measurements in 193 of the original study participants and found no differences between the high and standard titer vaccine recipients or between the sexes.

The Sudan study was unique among the larger high titer vaccine trials since each child was visited twice every month for 3 years after vaccination to evaluate the possible benefits of the high titer vaccines. We were able to use these data to look for evidence of possible harm (morbidity) associated with the high titer vaccines. We analyzed the data from more than 40,000 home visits and could find no evidence of a harmful effect of the high titer vaccines in either boys or girls.

Although the use of the high titer vaccines was suspended as soon as evidence of increased mortality was presented (1993), the data generated in this study are reassuring that no on-going harm is associated with the more than 20 million doses of these high titer vaccines distributed throughout the world. These data establish that the immunologic injury associated with high titer vaccines does not persist for more than 3-4 years after vaccination. In addition, this study is the first to demonstrate that recipients of the high titer vaccines were not 'sickly' after exposure to the vaccine. This observation suggests that individual differences in the children receiving these vaccines may have contributed to the observed increase in the risk of death.

3. **Overview of Grant-Supported Activities**

Since the children enrolled in Phase I were growing up, there was considerable pressure to collect as many samples as possible as quickly as possible in the summer of 1994. A graduate student (Fred Bertley) was assigned to the project and worked closely with Dr Salah during the summer and early fall (1994) to collect specimens from 193 children (see Section 4 for details of selection). The specimens were processed at the University of Khartoum in the laboratory of Dr Hashim Ghalib and frozen for transport to Canada. These specimens were hand-carried back to Canada and have been extensively studied as Fred Bertley's immunology MSc thesis project (Section 5). On the basis of this work, Mr Bertley has submitted his application to continue this line of study as a PhD. The central findings of this study have been submitted in abstract form to the American Society of Tropical Medicine (December 1996) and the first manuscript is in preparation. Dr Micheal Libman visited The Sudan in late 1994 to help the field team organize the mass of epidemiological data collected in Phases I and II of the study. This morbidity data s now in the final stages of analysis (See Section 6 - anticipated submission of manuscript - August 1996). Dr Ali, a junior faculty member of the University of Khartoum, was able to attend the Summer Epidemiology Program at McGill University in 1995. Finally, the clinical material collected in Phase I of the Sudan study was transferred to Canada and has already been useful to address questions of measles immunology un-related to the central question of high titer vaccination (abstract presented at 1996 Canadian Society for Immunology - manuscript in preparation).

It should be emphasized that although the grant was awarded for a 12 month period, we have worked full-time with the samples collected for more than 18 months now. We have only just completed some of the more sophisticated analyses.

4. **Selection of Children for Study in Phase II**

I would like to acknowledge the assistance of Dr Theresa Gyorkos with this part of the work (Clinical Epidemiology - McGill). She has been an invaluable member of Fred Bertley's thesis committee.

Phase I of the Sudanese HT measles vaccine trial was conducted between 1989-92. Children were recruited from 14 villages of the Umdawanband and Essailat rural councils, approximately 50 kilometers from Khartoum. These villages range in size from 800 to 5900 residents and there are no significant socio-economic, environmental or nutritional differences between the children in the different villages. Over a ten-month period, 510 five-month old infants were enrolled in the vaccine study. These children were block randomized to receive one of three vaccine regimens:

1) Edmonston-Zagreb HT at 5 months + Meningococcal vaccine at 9 months
2) Connaught HT at 5 months + Schwarz standard titer at 9 months
3) Meningococcal vaccine at 5 months + Schwarz standard titer at 9 months

There were no significant differences between the vaccine groups at the time of recruitment to the initial trial or at the initiation of Phase II. In Phase II, 6 of the 14 villages were chosen at random and an attempt was made to recruit all of the children in these villages. This was statistically permissible since there were no relevant differences between the original study villages. We were able to recruit 193 children of the 280 originally enrolled in the Phase I vaccine trial (69%; See Table 1). All of the vaccine groups are well represented in this sample (Table 2).

Table 1: Village Size and Number of Participants in Primary and Follow-Up Studies

Village	Population	#Children in Vaccine Study	#Children in Immunologic Study	% of Vaccinees
Umdawanban	4800	96	--	
Hawiela	800	11	--	
Essailat	5900	106	88	83
Hasonab	1250	15	--	
Hamda West	800	14	--	
El Hashid/	1350	41	23	57
Abuzayd	1550	41	24	58
Baknab	1000	22	--	
Fadnia	950	27	19	70
Sheikh	1800	37	27	73
Mustafa Bambonad	1700	28	12	43
Mahab	1700	46	--	
Kutrang Kutang	2250	22	--	
Mahab Tayba	550	4	--	
Total (Phase I)	26,400	510		
Total (Phase II)		280	193	69%

Table 2: Distribution of Phase II Samples by Vaccine Group and Gender

Males			Females			Total
EZ	CN	Men	EZ	CN	Men	
41	28	33	32	33	26	193

EZ = Edmonston Zagreb
Men = Meningococcal (placebo)
CN = Connaught

5. Immunologic Data

Since measles continues to be a major killing infection in Sudanese children and the children in the original trial were vaccinated at an early age, we used a range of assays to evaluate both humoral and cellular components of measles specific immunity (Section 5.1). However, the reports of excess mortality in HT recipients implicated non-measles pathogens as the causal agents. We therefore measured humoral and cellular immunity to a range of other childhood pathogens as well (Section 5.2).

5.1 Measles-specific Immune Responses 4-5 Years after Vaccination

5.1.1 Humoral Responses to Measles Antigens

5.1.1.2 Total IgG ELISA

Total anti-measles antibody was evaluated by an ELISA developed in our laboratory. The results of this assay are reported as milli-international units based upon comparison with the WHO reference sera (Table 3). Levels > 200 mIU are correlated with protection against infection. Virtually all of the children had protective levels of anti-measles antibodies. While this finding was reassuring at one level, it was also surprising since 12% of the EZ group had been seronegative three months after vaccination. Although no measles cases were reported in the study children during the 3 year morbidity follow-up (see Section 5), this observation suggests that wild-type measles virus was circulating unrecognized in the study communities.

As expected, the children vaccinated at 9 months of age had the highest titers of anti-measles antibody. It was somewhat surprising that the children in the Connaught group (vaccinated at 6 months and again at 9 months) did not have higher titers although early vaccination has previously been reported to diminish the antibody response to booster immunizations. Priming of these children for cellular rather than humoral responses may explain this observation (see Section 8). Overall, girls in the high titer groups tended to have lower IgG levels than their male peers but these differences did not reach statistical significance (Table 4).

Table 3

Total Measles-Specific IgG (ELISA)

Vaccine Group	(n)	IgG Titer
Control	(61)	276 ± 29
Edmonston-Zagreb	(73)	213 ±26
Connaught	(59)	240 ± 35

Table 4

Total Measles-Specific IgG (ELISA)
Grouped by Gender

Vaccine Group	(n)	IgG Titer Male	IgG Titer Female	p
Control	(61)	235 ± 30	315 ± 48	.17
Edmonston-Zagreb	(73)	240 ± 33	180 ± 42	.26
Connaught	(59)	288 ± 51	178 ± 43	.12

5.1.1.2 Neutralizing Antibodies by Syncytium Inhibition

Neutralizing antibody assays are more time consuming and expensive than standard ELISAs but are the 'gold standard' for measles serology. Syncytium inhibition titers ≥ 32 are thought to be a reliable indication of protection against measles.

The neutralizing antibody results were essentially identical to those generated by ELISA (Table 5). There were also no differences in IgG subclass distribution or IgG avidity between the groups or the sexes (data not shown).

Table 5

Measles-Specific Neutralizing Antibody (SIA)
Grouped by Vaccine and Gender

Vaccine Group	(n)	SIA Titer Male	SIA Titer Female	SIA Titer All
Control	(61)	183 ± 30	432 ± 101	310 ± 56
Edmonston-Zagreb	(73)	225 ± 55	190 ± 41*	210 ± 36
Connaught	(59)	247 ± 44	211 ± 45	231 ± 31

* p < .03 vs Control

5.1.2 Cellular Response to Measles Antigens

Cellular responses to measles antigens have never been measured before in children in the developing world. Our laboratory has recently developed a lymphoproliferative assay which made this possible in the Sudan HT children. The results of this assay are reported as stimulation indices (SI) which is an indication of antigen-specific T cell proliferation (divided by non-specific proliferative activity). SI ≥ 3 are generally considered to indicate significant lymphoproliferative activity. The measurement of cellular responses was particularly important in these children because previous studies of immune function in HT recipients had suggested defects in cellular rather than humoral

immunity. Since several different vaccines were used and 'effective' cell-mediated immunity (CMI) would presumably be directed against wild-type measles antigens, we tested the children's' blood cells using 3 different measles antigens: a wild-type antigen (CHI-1), a partially attenuated vaccine strain (Edmonston B) and a fully attenuated vaccine strain (Connaught).

The lymphoproliferative responses to the three measles antigen preparations were similar and only the results of the challenge with wild type virus are presented (Figure 1). The Sudanese children generally had low proliferative responses compared with Canadian children 4-5 years after vaccination (mean Canadian SI 8-10; $p < .001$). The Connaught group tended to have the highest proliferative responses although the differences did not reach statistical significance. There were no significant differences between the sexes (Figure 2).

Figure 1

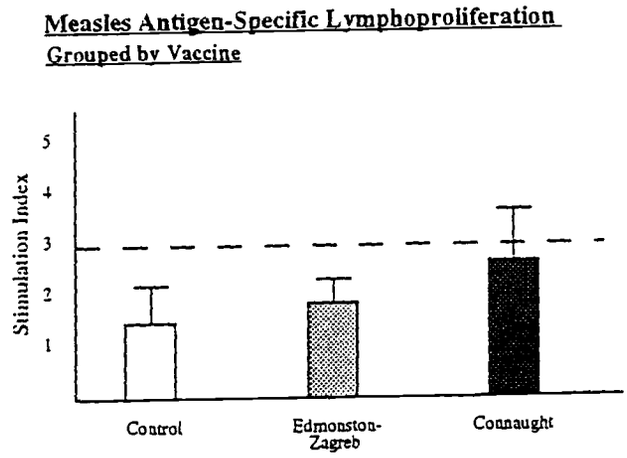
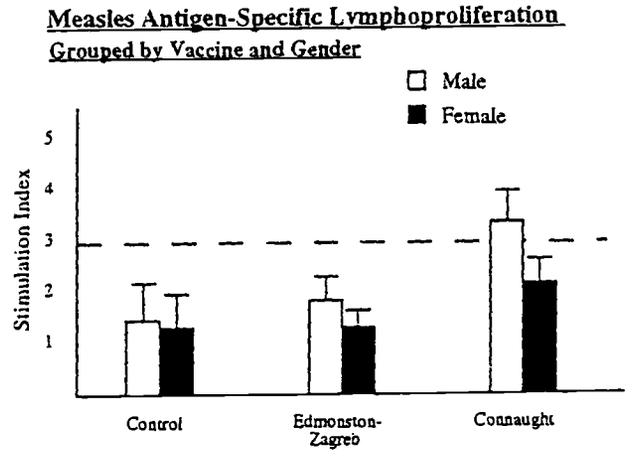


Figure 2



5.2 Immune Responses to Non-measles Pathogens at 4-5 Years of Age

5.2.1 Humoral Responses to Non-measles Pathogens

ELISA assays were developed in our laboratory to measure specific antibody responses to four of the major childhood pathogens: *Clostridium tetani, Corynebacterium diphtheria, Bordatella pertussis and Haemophilus influenza.* International standard sera are available for the first two organisms and were used to standardize the assays in milli-international units (mIU). A local standard was used to control the other assays and results are reported in arbitrary units (U). Vaccination histories for the study children (other than measles) were not considered to be reliable and there was no active program of primary immunization in the study region. Therefore, the immunity to non-measles pathogens in the study children is considered to be predominantly 'natural'.

All of the study children had protective levels of antibodies directed against tetanus (> 0.01 mIU) and diphtheria toxins (> mIU) .There were no statistically significant differences between high and standard titer recipients for any of the serologies (Table 6). Female HT EZ recipients tended to have more antibody directed against tetanus toxoid and pertussis while male HT EZ recipients generally had less antibody directed against pretties (Table 7).

Table 6

Antibody Titers against Non-Measles Pathogens
Grouped by Vaccine

	Titer		
Antigen	Control	Edmonston-Zagreb	Connaught
Tetanus toxoid	58 ± 7	63 ± 5	64 ± 8
Diphtheria toxoid	12 ± 2	13 ± 1.4	15 ± 1.5
Bordatella Pertussis	25 ± 2	27 ± 1.5	28 ± 1.5
Haemophilus influenza	12 ± 2	14 ± 2	11.5 ± 1

Table 7

Antibody Titers against Non-Measles Pathogens
Grouped by Vaccine & Gender

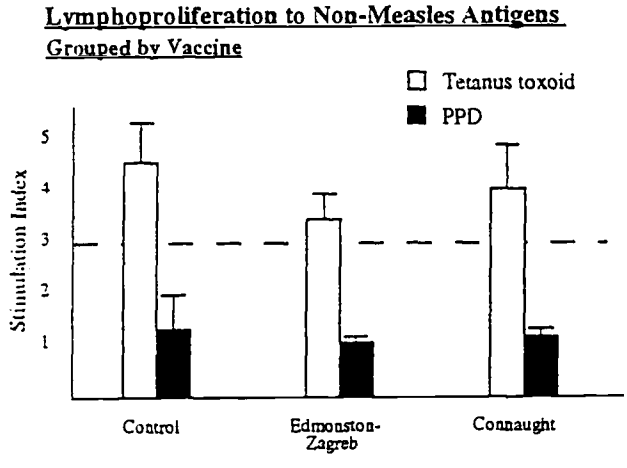
	Titer					
Antigen	Control		Edmonston-Zagreb		Connaught	
	male	female	male	female	male	female
Tetanus toxoid	74 ± 9	54 ± 10	64 ± 8	62 ± 7	71 ± 9	40 ± 9
Diphtheria toxoid	15 ± 2	14.5 ± 2	13 ± 2	14 ± 2	13 ± 2	11 ± 2.5
Bordatella Pertussis	29 ± 2	26 ± 2	23 ± 2	31 ± 2	28 ± 2.5	20 ± 3
Haemophilus influenza	11 ± 2	12.5 ± 2	13 ± 2	15.5 ± 2.5	11 ± 3	13.5 ± 3

5.2.2 Cellular Responses to Non-measles Pathogens

CMI has only recently been recognized as an important (if not critical) element of the immune response to many childhood pathogens. We developed lymphoproliferative assays for *Clostridium tetani* and purified protein derivative (PPD; an indication of anti-tuberculosis activity). Again, results are expressed as stimulation indices and SI ≥ 3 are considered to be significant.

In general, children responded well to tetanus toxoid and poorly to PPD. There were no statistically significant differences between the groups or the sexes for either of these cellular responses (Figure 3).

Figure 3



5.3 Other Immunological Parameters

In order to look for more subtle immunologic differences in the HT recipients, we have recently completed data collection for an extensive FACS-based, phenotypic analysis of a subset of the children (n = 75; twenty-five children from each vaccine group). We used a panel of 30 monoclonal antibodies directed against peripheral blood mononuclear cells subsets ($CD4^+$ and $CD8^+$ T cells, B cells, monocytes, NK cells), activation markers and co-stimulatory molecules (Table 8).

Table 8

Monoclonal Antibodies used for FACS Analysis

CD4 T Cells	CD45RA	naive cell marker	B Cells	CD23	activation marker
	CD45RO	memory cell marker		ICAM-1	adhesion molecule
	CD45RB	transition phenotype		CD 80	costimulatory molecule
	CD75	high affinity IL-2 receptor		CD 86	costimultory molecule
	CD25	low affinity IL-2 receptor		CD25	low affinity IL-2 receptor
	HLA-DR	MHC class II			
	CD30	Th2 phenotype marker			
	CD28	costimulatory molecule	Monocytes	ICAM-1	adhesion molecule
	CD8	CD4/CD8 ratio		CD 80	costimulatory molecule
	CD71	transferrin receptor		CD 86	costimultory molecule
	LFA	adhesion molecule			
CD8 T Cells	CD38	activation marker	NK Cells	CD38	activation marker
	CD69	activation marker		CD69	activation marker
	CD75	high affinity IL-2 receptor			
	CD25	low affinity IL-2 receptor			
	HLA-DR	MHC class II			
	CD4	CD4/CD8 ratio			
	LFA	adhesion molecule			

Although we have not yet completed the final analysis, it does not appear that there are any major differences between the vaccine groups or between the sexes. Minor differences between groups are present (eg: high NK cell expression of the activation marker CD69 in HT EZ recipients; $p < .04$ vs control) but there is no consistent pattern of either activation or suppression in the HT recipients.

6. **Morbidity Follow-up**

Phase I of the study included a regular (bi-monthly) follow-up to assess the anticipated benefits of the high titer vaccines (eg: fewer days sick, economic benefits). The regular visits were performed by village healthcare workers trained by the project. Any child who was found to be ill was visited by one of the study pediatricians within a week (generally within 3-4 days). It should be emphasized that these data were collected prospectively to screen for potential benefit of the HT vaccines. Like all of the other HT vaccine trials, Phase I was not designed to look for long-term adverse events. The analysis we have performed is therefore a 'retro-fit' of the available data to address an unanticipated outcome.

A tremendous amount of work on Dr Libman's part can be summarized with the simple statement: There is no evidence that HT vaccination influences early childhood morbidity. Measured outcomes included the number of days ill per month, the number of illnesses per month (Figures 4 & 5), the number of days with fever, cough or diarrhea per month, the number of investigator visits with fever, cough or diarrhea (Figures 6 & 7).

Figure 4

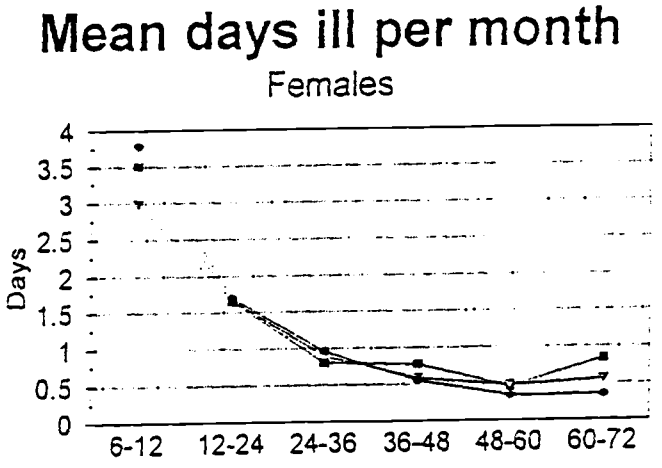


Figure 5

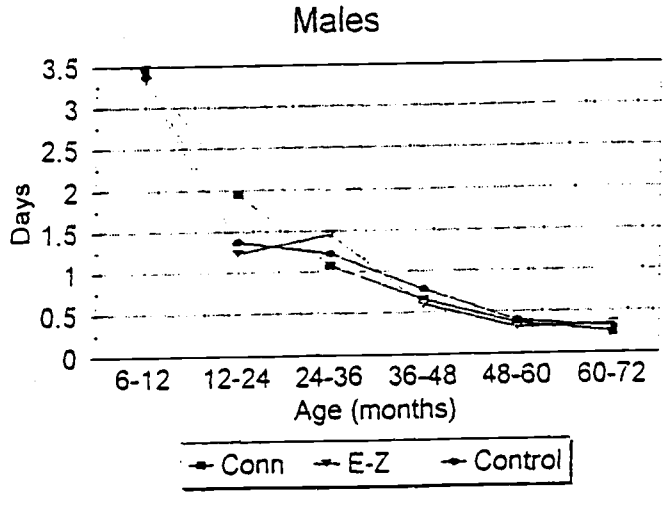


Figure 6

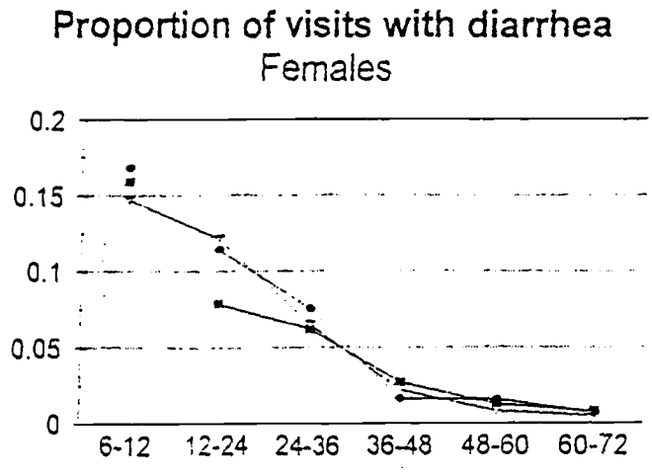
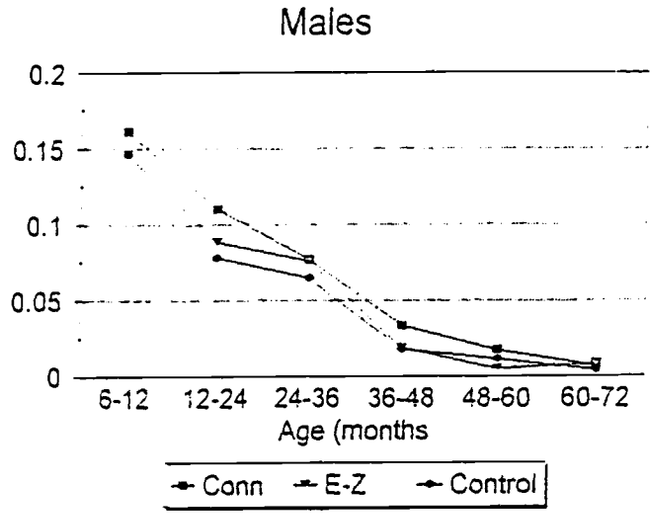


Figure 7



This is the most convincing data generated to date that the impact of measles HT vaccination on infant mortality was not due to cumulative morbidity. These data strongly suggest that the HT effect was idiosyncratic in nature.

7. **Possible Economic Analysis**

We have performed a preliminary analysis of the economic data collected during the periodic follow-up in Phase I with essentially negative results. This is not surprising since the HT vaccine recipients in The Sudan appear to have neither benefited nor suffered significantly compared with their peers who received standard titer vaccination.

8. **Other Scientific Uses of the Sudanese Data**

A large amount of clinical material and epidemiological information was collected during Phase I and Phase II of the Sudan study. We have spent a good deal of time organizing this material/data so that our study goals could be accomplished but also to permit other, unanticipated uses. To date, we have identified one very exciting

opportunity to study basic measles vaccine immunology using the Sudan data set. That is: whether or not early measles vaccination can immunologically 'prime' individuals for a better or different immune response. Our preliminary analysis of this question is outlined below and was the subject of an abstract presented at the Canadian Immunological Society meeting in Chanteclerc, Quebec (March 1996).

8.1 Exposure to Low Dose Measles Antigen may Bias towards Cellular Immunity

The presence of maternal antibodies makes measles vaccination in children < 9 months of age very difficult. Vaccination with the standard dose vaccine prior to 9 months results in poor seroconversion rates. Furthermore, the antibody response to revaccination in children first vaccinated at a time when maternal antibodies were present is weaker than that observed in children who are vaccinated only once after 9 months of age. Successful vaccination has conventionally been defined in terms of the humoral response alone. However, the immune correlates of protection against measles are not known and no attention has been given to measles specific cell mediated immunity (CMI). Since measles is an intracellular pathogen, CMI may be a important element in controlling infection and the long-term maintenance of protection. Indeed, patients with aggammaglobulemia recover uneventfully from measles infection, while patients with defective CMI (e.g. HIV) suffer 50 - 100% mortality from measles . Recent work has demonstrated that the dose of antigen can play an important role in determining the type of immune response elicited (e.g. humoral or cellular predominance). *In vivo* animal models have shown that low doses of an antigen can prime the immune system for a cellular response upon subsequent antigenic challenge. Also, naive $CD4^+$ T cells are susceptible to biasing towards an antigen-specific Th1 responses when exposed to intermediate doses of a peptides. Although these experiments raise the possibility of directing the induction of protective antigen-specific cellular responses, no similar human data has been described. The Sudanese vaccine study allows the hypothesis that early exposure of children to measles vaccine (i.e. in the presence of maternal antibodies) might favor the production of measles specific CMI to be explored.

In the initial vaccine study in The Sudan, one third of the children received Connaught vaccine ($10^{4.6}$ pfu) at 5 months and Schwarz standard titer vaccine (10^3 pfu) at 9 months of age (Connaught; n = 170). The control children received menningoccocal vaccination at 5 months and standard Schwarz vaccine at 9 months (Control; n=170). In Phase II of the study (4-5 years after vaccination), plasma and peripheral blood mononuclear cells were collected from 59 Connaught and 61 Control vaccine recipients.

Since Connaught children received their initial vaccination at 5 months of age, it was predictable that a proportion would "fail" vaccination (i.e. fail to develop significant antibody titers). The hypothesis of low dose antigen biasingtowards a Th1 response could therefore be tested by comparing the long-term cellular and humoral responses generated by Connaught vaccine failures (n = 12/59; 20%) with the immune responses generated by Control children (n = 61).

8.1.1 Humoral Responses

The mean neutralizing antibody titer for Group 1 children tended to be lower than that in Group 2 (SIA 178.739.3 vs 309.855.7) but this difference did not reach statistical significance (p=.22).

8.1.2 Cellular Responses

In contrast, Group 1 children had significantly higher cellular responses than the Group 2 children (SI 3.1 vs 1.4; $p < .04$).

These data suggest strongly that the early 'ineffective' exposure to measles antigens (in terms of antibody production) biased the Group 1 children towards cellular rather than humoral responses upon subsequent exposure to measles antigens. These data may have significant implications for measles vaccination strategies throughout the world.